Exhibit 2 - Report of Second Clinical Trial

Acambis

CLINICAL STUDY REPORT

Phase 1, Dose Escalation, Safety and Immunogenicity of Two New Attenuated Vaccine Strains for Enterotoxigenic *E. coli* in volunteers

Outpatient study

(Protocol# VTU983)

BB-IND#: 7922

Sponsor: David A. Sack, M.D.
A. Louis Bourgeois, Ph.D., MPH
The Johns Hopkins University Bloomberg School of Public Health

Financed by:
Acambis, Inc.
38 Sidney Street
Cambridge, MA 02139
Tel: 617.494.1339
Fax: 617.494.1741

Investigators:
David A. Sack, M.D. and A. Louis Bourgeois, Ph.D., MPH, The Johns Hopkins University Bloomberg School of Public Health, Vaccine Testing Unit, 550 N. Broadway, Suite 1001, Baltimore, MD 21205, USA.

TABLE OF CONTENTS

LIST OF TABLES IN TEXT

LIST OF FIGURES IN TEXT

GLOSSARY OF ABBREVIATIONS

Abbreviation	Definition
AE	Adverse Event
ALS	Antibody from lymphocyte supernatant
ASC	Antibody secreting cells
CTB	Cholera toxin B subunit
CFA	Colonization factor antigen
CFR	Code of Federal Regulations
CMI	Cell Mediated Immunity
CRF	Case Report Form
CT	Cholera Toxin
E.COLI	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *E. coli*
FDA	Food and Drug Administration
GCP	Good Clinical Practice
GCRC	General Clinical Research Center
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IRB	Institutional Review Board
LT	Heat-labile enterotoxin from *E. coli*
ML	Milliliters
NMRC	Naval Medical Research Center
PBML	Peripheral blood mononuclear lymphocytes
SAE	Serious Adverse Event
ST	Heat-stable toxin
μg	Microgram
VTU	Vaccine testing unit

1 SYNOPSIS

Name of Sponsor / Company: David Sack, M.D. and transferred to A. Louis Bourgeois, Ph.D., MPH (Johns Hopkins University Bloomberg School Of Public Health) (BSPH) / Acambis, Inc.	**Individual Study Table Referring to Part of the Dossier** **Volume:**	**(For National Authority Use only)**
Investigational product: Enterotoxigenic *E. coli* (ETEC) vaccine PTL-ETEC-002 and PTL-ETEC-003		
Active ingredient: Live fresh washed ETEC bacteria	**Page:**	

Title: Phase 1, Dose Escalation, Safety and Immunogenicity of Two New Attenuated Vaccine Strains for Enterotoxigenic *E. coli* in volunteers
Investigators: David Sack, M.D. (Principal Investigator), A. Louis Bourgeois, Ph.D., MPH (Principal Investigator), and Robin McKenzie, M.D., Fayette Engstrom, M.D., Janet Shimko, RN, MSN, and Eric Hall, Ph.D. (Sub-Investigators)
Study Center: Outpatient clinical studies carried out at the Vaccine Testing Unit (VTU), BSPH, 550 N. Broadway ST. Baltimore, MD
Analytical site: Clinical Laboratory - Johns Hopkins Hospital and Research Microbiology and Immunology – Department of International Health, BSPH and in the Naval Medical Research Center (NMRC), Silver Spring, MD.

Study Period of Clinical Phase: **Date first volunteer admitted for enrollment (vaccination):** November 11, 1999 **Date first volunteer enrolled (vaccinated):** November 12, 1999 **Date last volunteer completed:** February 18, 2000 (28 days post last vaccination – 1/21/00)	**Clinical Phase**: I

Objectives:

- To extend the finds of the inpatient study by further assessing the safety and immunogenicity of both candidate vaccine constructs in outpatients using a randomised, double-blind, placebo-controlled study design.
- To evaluate the comparative safety and immunogenicity of 1 versus 2 oral doses of both vaccine constructs.
- Select the most tolerable and immunogenic vaccine construct for further development as a candidate ETEC vaccine.

Methodology: Single-center, double-blind, placebo controlled, outpatient, safety and immunogenicity study to evaluate two new attenuated strains of enterotoxigenic *E. coli* vaccine (PTL-ETEC-002 and PTL-ETEC-003). Vaccine was administered orally as two doses of a vaccine or one dose of a vaccine and one dose of placebo to eligible outpatient volunteers. All volunteers received at least one dose of vaccine, but some received the placebo as a first dose and others received the placebo as a second dose. A dose of vaccine consisted of 2 x 10^9 CFU. One dose of vaccine/placebo was given on Day 0 and the second dose given 10 days later on Day 10.

Number of subjects (*planned and analysed*): 38-40 planned, 40 volunteers were enrolled, 12 volunteers were to receive two doses of strain PTL-ETEC-002 (Group 1), 12 volunteers were to receive two doses of strain PTL-ETEC-003 (Group 2), and 16 volunteers were to receive one dose of vaccine (PTL-ETEC-002 or PTL-ETEC-003) and one dose of placebo in a crossover design (Groups 3, 4, 5 and 6). In each case the dose of the vaccine received was 2 x 10^9 bacteria. All 40 volunteers received the first dose of vaccine or placebo and 37 received the second dose. Of the 3 volunteers who missed the second dose, 2 were to have received a second dose of PTL002 or PTL003 and these were reassigned to the single dose vaccine group for data analyses, the third was to have received placebo.

Diagnosis and main criteria for inclusion: Healthy, non-immunocompromised, male or female outpatient volunteers, >18 or <50 years of age with none of the following: clinically significant medical history, physical examination, or screening laboratory examinations (complete blood count with differential, blood chemistry, urinalysis), negative serologies for HbsAg, HCV, HIV, negative urine HCG the day before immunization (women only), and volunteers over the age of 40 with a normal EKG. Volunteers were required to complete a training session, provide written informed consent, and demonstrate comprehension of the protocol procedures and knowledge of diarrhea, ETEC bacteria by passing a written examination. Volunteers were excluded from the study if they had a chronic illness, regular use of laxatives or abnormal stool pattern, if they travelled to a developing country within 5 years, if they previously participated in an ETEC study, if antibiotics were used within 7 days of vaccination, if they worked in child care, if they had a child under 5 years of age at home, if they had a person at home who was immunocompromised, if they had direct contact with volunteers in the health care setting, or if they were a food handler. In addition the subjects were instructed to avoid becoming pregnant during the study and to avoid swimming in unchlorinated waters during the duration of the study.

Test product, dose and mode of administration, batch number: Lyophilized cultures of PTL-ETEC-002 and PTL-ETEC-003 were prepared for Acambis Research Ltd. by PHLS Central Public Health Laboratory and these were sent to the PI at Johns Hopkins University who in turn produced frozen working cell banks. The final vaccine was comprised of freshly grown vaccine cultures derived from the working cell banks. Sequential groups of volunteers were to receive oral doses of 2x10^9 CFU, administered by mouth, in 200 ml of CeraVacx (a rice-based buffer containing 2 grams of sodium bicarbonate, 0.5 grams of trisodium citrate in addition to 7 grams of a proprietary rice syrup) containing the vaccine. No buffering of the stomach pH was done prior to vaccine/placebo dosing. Volunteers randomized to receive placebo, received 200 ml of CeraVacx™ only.

Reference product: Placebo: CeraVacx™ buffer (Cera Products Inc., Jessup, MD.)

Duration of study drug treatment: Oral administration of one dose level of vaccine, at 2 x 10^9 CFU, two-dose regimen, first dose administered on Day 0 and second dose administered on Day 10. Placebo dosing, where appropriate, was administered on the same time schedule.

Criteria for Evaluation:

Safety: Reactogenicity was ascertained by analysing documented signs and symptoms of illness. A symptom diary was maintained for seven (7) days after each dose. Subjects were also asked to return to the VTU for an assessment of adverse events prior to the first dose vaccination, Days 3, 7, and 10 after the first dose and Days 3, 7, 10, 14 and 28 after the second dose. Stool was collected on Day 0 and then on Days 3, 7 and 10 after the first dose and Days 3, 7, 10 14 and 28 after the second dose to detect excretion of the vaccine strain. If the vaccine strain was still present in the stool 14 days after the second dose, Ciprofloxacin was to be administered (500 mg BID) and at least two follow-up stool specimens were to be collected to verify that the vaccine strain has been cleared.

Immunogenicity: The immunogenicity of the two vaccines were evaluated by measuring anti-CFA/II specific IgA and IgG Antibody secreting cells (ASC) and serum antibody responses post-immunization. Blood for serum and lymphocyte culture were collected on Days 0, 7 and 10 after the first dose and Days 7, 14 and 28 following the second dose. Urine and stools were collected for antibody determination on Days 0, 7 and 10 after the first dose and Days 7, 14 and 28 after the second dose.

Statistical methods: Adverse events were summarized by frequency of occurrence, number of subjects experiencing adverse events, severity and relationship to investigational vaccine. The frequency of occurrence of adverse events was compared by Fisher's Exact test between placebo and vaccine recipients. Immune responses to the vaccines were determined qualitatively, the criteria for determining positive and negative responders were determined prior to unblinding and the assignment of positive and negative responders was also performed on blinded data. Immune response frequencies to CFA/II antigen in one and two dose recipients were compared by X^2 or Fisher's exact test.

Safety results: No serious vaccine related adverse events were reported. No clinically significant trends in adverse events, vital signs or screening clinical laboratory test were observed in regard to subject safety.

Forty-two (42) subjects were enrolled in the outpatient phase of the study. Two individuals withdrew before receiving vaccine. The remaining 40 subjects were randomly assigned to one of six groups to receive vaccine or placebo on Days 0 and 10. Twelve were assigned to receive two doses of PTL-ETEC-002 and 12 to receive two doses of PTL-ETEC-003. Four (4) were randomized into each of the single-dose groups to receive vaccine followed by placebo or vice versa. All volunteers received the first dose of vaccine or placebo and 37 received the second dose. The data available for all 40 subjects following one dose of vaccine, for 10 subjects following a second dose of PTL-ETEC-002, and for 11 subjects following a second dose of PTL-ETEC-003.

The overall incidences of general symptoms were not significantly different (Fisher's exact test) for subjects receiving either PTL-ETEC-002 or PTL-ETEC-003, compared to placebo recipients. There were no incidences of oral temperature greater that 100.4 °F. There were reports of illness; weakness, headache, light-headedness, muscle aches and chills but the percent of subjects in any one group experiencing these symptoms never exceeded 20%. Headache was the most common complaint with incidences of 17% - 19% after receiving placebo, PTL-ETEC-002, or PTL-ETEC-003. The incidences of symptoms were neither more nor less frequent after two doses in comparison with one dose.

Gastrointestinal symptoms associated with infections with wild type ETEC were not found more frequently in subjects receiving PTL-ETEC-002 or PTL-ETEC-003 compared with those receiving placebo or untreated. There were no reports of blood in stool or belching. There were less than 5 reported cases in the combined groups of decreased appetite, nausea, abdominal pain, gurgling stomach, pain in the rectum with defecation and vomiting. There were 15 reports of gas, 12 reports of cramping, 11 reports of unformed stool, and 6 reports of urgency of defecation. None of the incidences were statistically different after receipt of PTL-ETEC-002 or PTL-ETEC-003 compared to placebo or no treatment (Fisher's exact test). Only two episodes of diarrhea (>3 unformed or liquid stool during a 24 hr period) and one episode of vomiting were detected during the trial. The diarrhea occurred in 1 vaccine recipient (PTL-ETEC-003) and 1 placebo recipient, and the vomiting occurred in a placebo recipient.

Efficacy results:

Excretion of vaccine strains: Nine of 10 subjects receiving 2 doses of PTL-ETEC-002 had positive stool cultures after either 1 or 2 doses. Of the 10 subjects who only received a single dose of PTL-ETEC-002, either by design (i.e. received placebo as the other dose) or due to illness or drop-out, 8 subjects were stool culture positive on at least one occasion.

All 11 subjects receiving 2 doses of PTL-ETEC-003 were stool culture positive after either one or 2 doses of vaccine. Eight of the 9 subjects receiving a single dose of PTL-ETEC-003, either by design or due to illness or drop-out, had positive stool cultures.

The number of positive stool samples per subject receiving PTL-ETEC-002 ranged from 0 - 14 with a median of 1 and an average of 2.1. The number of positive stool samples per subject receiving PTL-ETEC-003 ranged from 0 - 19 with a median of 1 and an average of 4.6. The data indicate that the majority of recipients had either no or one positive stools. It should be noted that since subjects receiving 2 doses of vaccine are counted twice, once after the first dose and then again after the second dose, the majority of cases (6 of 9 for PTL-ETEC-002 and 3 of 4 for PTL-ETEC-003) with no positive stools occurred after receiving the first dose of active vaccine and the subjects were culture positive following the second dose of active vaccine.

Serology results: The antibody-secreting cell (ASC) response was determined in peripheral blood mononuclear lymphocytes (PBMLs) by comparing the number of ELISPOTS at baseline (Day 0) with results 7 days after vaccine administration (Days 7 or 17). An ASC response was defined as 1.3 or more spots per 10^6 cells 7 days after vaccination and, if the baseline value was positive, a doubling of the baseline value. PBMLs were also analyzed by antibody lymphocyte supernatant assay (ALS). Unstimulated PBMLs were cultured for 48 hours and the culture supernatant subjected to ELISA assay for CFA/II specific IgG or IgA. An ALS response was defined as a 2-fold or greater increase compared to the baseline sample.

All 40 of the volunteers provided blood samples at Day 0 and Day 7. As described above, one subject who received two doses of PTL-ETEC-002 did not return after the second vaccination. One assigned to receive PTL-ETEC-002/placebo missed the visit at Day 10, and one who received PTL-ETEC-003/placebo missed a single visit at Day 17. Therefore, PBMCs were available at baseline and 7 days after one dose of vaccine for all 40 subjects, 20 of who received PTL-ETEC-002 and 20, PTL-ETEC-003. In addition, PBMCs were available 7 days after a second dose of vaccine for 10 who received a second dose of PTL-ETEC-002 and 11 who received a second dose of PTL-ETEC-003.

The PTL-ETEC-003 vaccine construct was also superior to the PTL-ETEC-002 construct in its ability to induce both IgA and IgG serum antibody responses to CFA/II. This difference was most pronounced in the IgA-isotype. The cumulative proportion of volunteers exhibiting CFA/II-specific IgA seroconversions after one or two doses of vaccine was significantly higher in those subjects receiving the PTL-ETEC-003 vaccine compared to those given the PTL-ETEC-002 construct (35% vs. 0% respectively). Again, this difference between the two constructs was even more apparent when response frequencies were compared in those volunteers receiving only a single dose of either vaccine. Five of the 9 (56%) volunteers given a single dose of PTL-003 seroconverted to CFA/II, while none of the volunteers given a single dose of PTL-002 responded. Similar trends were apparent in the proportion of CFA/II-specific IgG seroconverters but these differences were not significant.

Conclusion: The overall incidences of general and gastrointestinal symptoms were not significantly different (Fisher's exact test) for subjects receiving either PTL-ETEC-002 or PTL-ETEC-003, compared to placebo recipients. Both attenuated strains, PTL-ETEC-002 and PTL-ETEC-003, were well tolerated with no significant general or gastrointestinal symptoms associated with the administration of either strain. The PTL-ETEC-003 construct was superior to the PTL-ETEC-002 construct in its ability to induce both mucosal and systemic immune responses to CFA/II. PTL-ETEC-003 also exhibited a more sustained intestinal colonization than PTL-ETEC-002. The anti-CFA/II immune responses induced by PTL-ETEC-003 were comparable after either one or two doses of vaccine; a booster immune response was not evident although a few more seroconvertors were identified after the second dose. The anti-CFA/II immune response induced by PTL-ETEC-003 was comparable to those induced by other candidate ETEC vaccines in clinical trials.

These data indicate that the PTL-ETEC-003 vaccine construction is safe and forms the basis for further evaluation of PTL-ETEC-003 in a "proof-of-concept" volunteer challenge study. A toxin-positive ETEC challenge strain (E24377A) homologous in CFA makeup to PTL-ETEC-003 will be validated in an inpatient dose response study (this clinical trial is currently ongoing under BB-IND-9895) and then used to test the protective efficacy of vaccination with PTL-ETEC-003.

REPORT SIGNATURES

Our signature(s) below confirm the accuracy and content of the data contained within this report and our respective analyses and summaries thereof:

Investigator:
A. Louis Bourgeois, Ph.D., MPH

_____ _____
Signature Date

Medical Monitor:
William B. Greenough, M.D.

_____ _____
Signature Date

Author of Report:
Maria T. Berkheimer, M.S.
The Total Approach Inc.

_____ _____
Signature Date

Acambis:
Cynthia K. Lee, Ph. D.
Acambis Inc.

_____ _____
Signature Date

2 ETHICS

2.1 Institutional Review Board (IRB)

Prior to implementation, the study protocol was approved in writing, by the IRB of the Johns Hopkins University School of Medicine or Johns Hopkins Hospital. All subject-related procedures were carried out at the Vaccine Testing Unit's Outpatient Clinic, Johns Hopkins University Bloomberg School of Public Health (BSPH), Baltimore, MD.

IRB membership was maintained according to federal guidelines set forth in CFR Part 56. Details of the constitution of the IRB, including the names of their Chairs, are held on file at the Vaccine Testing Unit (VTU), The Johns Hopkins University School of Medicine. For a copy of the IRB approval details refer to **Appendix 12.4**.

This study was conducted under an IND (BB-IND#7922, Serial #001) held by David A. Sack, M.D. and later transferred along with investigator responsibilities to A. Louis Bourgeois, Ph.D., MPH, BSPH. The study was financed by Acambis Research Limited previously known as Peptide Therapeutics Limited.

2.2 Ethical Conduct of the Study

The study was carried out in accordance with the ethical principles outlined in the Declaration of Helsinki, 1964 and subsequent amendments.

2.3 Subject Information and Consent

In response to advertisements published in local papers (see example in **Appendix 12.4**), subjects interested in participation contacted the VTU and were invited to attend a briefing at the VTU at which the study was outlined. Written information/consent forms were given to the subject to study. For participants at VTU, there were separate consent forms for the collection of screening blood samples and for study participation. Examples of all consent forms are given in **Appendix 12.2**. Subjects interested in participation were invited back to VTU for the collection of screening samples. Witnessed written informed consent was obtained by study personnel, prior to any study-related procedure. Enrollment to the trial took place a few days prior to, or on the day of the first vaccination. For eligible subjects, enrollment comprised a final briefing by one of the study personnel, and a written comprehension test (in which subjects were required to score at least 70%).

3 INVESTIGATORS AND STUDY ADMINISTRATIVE STRUCTURE

The following key personnel from the VTU were involved in the management of the inpatient study and the subjects enrolled:

Medical Monitor: William D. Greenough M.D.

Principal Investigator: David Sack, M.D. and then A. Louis Bourgeois, Ph.D., MPH

Sub-investigators: Robin McKenzie, M.D., Fayette Engstrom, M.D., Janet Shimko, RN, MSN, and Eric Hall, Ph.D.

Statistician: No formal statistics performed. Data listings and tabulations were compiled.

Study Coordinator: Patricia Maples, RN

Study Nurse: Patricia Maples, RN

Curricula vitae for key personnel are located in **Appendix 12.5**.

The Study Drug was administered by Robin McKenzie, M.D. and Fayette Engstrom, M.D.

Johns Hopkins Hospital Laboratory, 600 Wolfe Street, Baltimore, MD 21205 performed clinical chemistry and routine hematology assays.

The following personnel were responsible for the bacteriological and immunological assays: A. Louis Bourgeois, Ph.D., MPH; Eric Hall, Ph.D.; H. S. Chang, Ph.D.; J. Kyle, MPH; and George Gomes.

Management of the clinical data (i.e. all data with the exception of those derived from the bacteriological and immunological assays) was carried out by Vaccine Testing Unit: Robin McKenzie, M.D., Janet Shimko, RN, P. Maples, RN, and A. Louis Bourgeois, PhD., MPH.

4 INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is the major etiological agent associated with traveller's diarrhea in many parts of the developing world and is a major cause of morbidity in both military and civilian travellers to these regions[1]. It also causes up to 380,000 deaths in infants and young children in endemic regions.

There is currently no licensed vaccine available for the prevention of ETEC disease, although there is a candidate vaccine being developed by Powderject, which is currently undergoing phase III evaluation. This consists of an inactivated whole cell preparation of five different ETEC strains, combined with recombinant cholera toxin B subunit (CT-B), which is administered as two oral doses[2,3,4,5].

The vaccines tested in this study consisted of live attenuated strains of ETEC for oral delivery. Similar live attenuated bacterial vaccines have been developed against *Salmonella typhi* and *Vibrio cholera*. Live attenuated ETEC organisms colonize the intestinal mucosa of vaccinees, providing prolonged exposure to antigen, and will

avoid the need for the addition of exogenous adjuvant. It is hoped that a single dose of vaccine will prove to be effective.

ETEC pathogenicity is well understood; fimbrial Colonisation Factors (CFA) mediate adherence to the surface of the intestinal epithelium where the bacteria secrete enterotoxins, which are responsible for the debilitating watery diarrhea. Protective immunity requires both a secretory IgA response against the CFA to block adherence and toxin neutralising antibodies[6,7,8,9].

A spontaneous toxin deletion mutant of a diarrheagenic ETEC strain (E1392/75/2A) had previously been isolated and tested in phase I studies as a potential vaccine[8]. This is a CS1, CS3 expressing CFA/II strain of the O6:H16 serotype. While providing significant protection against challenge in volunteers, it still caused low-grade diarrhea in 15% of recipients. To further attenuate this strain, two deletion mutations were introduced into the chromosome of E1392/75/2A. The first strain (PTL-ETEC-002) is deleted in *aroC* and *ompR* genes and the second strain (PTL-ETEC-003) is deleted in *aroC, ompC* and *ompF*. *AroC* is the gene encoding chorismate synthase in the aromatic amino acid biosynthetic pathway. *OmpR* encodes a regulatory protein which controls the inverse regulation of *ompC* and *ompF*, encoding outer membrane porins expressed at high and low osmotic pressure, and certain other genes including those responsible for the expression of Vi antigen in *S. typhi*. Phenotypically both sets of mutations are expected to reduce the ability of the organism to adapt to the conditions in the human digestive tract, attenuating its ability to colonise and cause disease.

5 STUDY OBJECTIVES

To extend the finds of the inpatient study by further assessing the safety and immunogenicity of both candidate vaccine constructs in outpatients using a randomised, double-blind, placebo-controlled study design.

To evaluate the comparative safety and immunogenicity of 1 vs. 2 oral doses of both vaccine constructs. The immunogenicity assessment will be based on the ability of either vaccine construct and/or dosing regimen to induce anti-CFA/II specific immune responses in volunteers.

Select the most tolerable and immunogenic vaccine construct for further development as a candidate ETEC vaccine.

6 INVESTIGATIONAL PLAN

6.1 Overall Study Design and Plan-Description

The trial was designed as a 38-40 subject, single-center, double-blind, placebo controlled, outpatient, safety and immunogenicity study to evaluate two new attenuated strains of enterotoxigenic *E. coli* vaccine (PTL-ETEC-002 and PTL-ETEC-003). Vaccine was administered orally as two doses of a vaccine or one dose of a vaccine and one dose of placebo to eligible outpatient volunteers. All volunteers will receive at least one dose of vaccine, but some will receive the

placebo as a first dose and others will receive the placebo as a second dose. A dose of vaccine will consist of 2 x 10^9 CFU; a placebo dose will consist of CeraVacx buffer only. One dose was given on Day 0 and the second dose given 10 days later on Day 10. The protocol and the case report form are attached in **Appendices 12.1** and **12.3** respectively.

6.2 Discussion of Study Design

Since the primary objective of the trial was to evaluate the safety of two new attenuated vaccine strains, and an inpatient study established safety, a double-blind, placebo-controlled study design was deemed appropriate.

Fecal excretion and immunological parameters would be evaluated by dose group. Vaccine was administered on Day 0 to eligible outpatient volunteers and then a second dose was given 10 days later on Day 10. Thirty-eight to 40 planned subjects, 40 volunteers were enrolled. Twelve (12) volunteers were to receive two doses of strain PTL-ETEC-002 (Group 1), 12 volunteers were to receive two doses of strain PTL-ETEC-003 (Group 2), and 16 volunteers were to receive one dose of vaccine (PTL-ETEC-002 or PTL-ETEC-003) and one dose of placebo in a crossover design (Groups 3, 4, 5 and 6). In each case the dose of the vaccine received was 2 x 10^9 bacteria.

6.3 Selection of Study Population

6.3.1 Inclusion Criteria

The following inclusion criteria were applied:

a. healthy, male or female outpatient volunteers, >18 or <50 years of age,
b. completed training on ETEC, diarrhea and protocol procedures,
c. demonstrate comprehension of the protocol procedures and knowledge of diarrhea, ETEC bacteria by passing a written examination, and
d. provide written informed consent.

6.3.2 Exclusion Criteria

The following exclusion criteria were applied:

a. chronic illness,
b. immunosuppressive condition,
c. positive serology for HbsAg, HCV, and/or HIV,
d. positive urine HCG the day before immunization (women only),
e. antibiotics used within 7 days of vaccination,
f. significant abnormality in screening laboratory examinations (complete blood count with differential, blood chemistry, urinalysis),
g. significant physical examination,
h. if they travelled to a developing country within 5 years,
i. if they previously participated in an ETEC study,

j. if they worked in child care,
k. if they had a child under 5 years of age at home,
l. if they had a person at home who was immunocompromised,
m. if they had direct contact with volunteers in the health care setting,
n. if they were a food handler,
o. regular use of laxatives or abnormal stool pattern, and
p. volunteers over the age of 40 with a abnormal ECG.

In addition the subjects were instructed to avoid becoming pregnant during the study and to avoid swimming in an unchlorinated pool during the duration of the study.

6.4 Removal of Subjects From Treatment or Assessment

Subjects were removed from the study if, in the opinion of the investigator, the health status of the subject warranted withdrawal (either through an adverse event or concurrent illness), there was significant non-compliance with the protocolled assessments or visits, or consent was withdrawn.

Where possible, follow-up assessments were conducted as protocolled, to the end of the appropriate treatment period in all subjects who were withdrawn.

Subjects who were withdrawn from the study were not replaced.

6.5 Treatments

6.5.1 Treatments Administered

For the six sequential groups, volunteers were to receive on Day 0 and/or on day 10 the oral administration of $2x10^9$ CFU with 200 ml of CeraVacx™ (a rice-based buffer containing 2 grams of sodium bicarbonate, 0.5 grams of trisodium citrate in addition to 7 grams of a proprietary rice syrup). No buffering of the stomach pH was done prior to vaccine/placebo dosing. Volunteers randomised to receive placebo were given 200 ml of CeraVacx™ buffer only.

6.5.2 Identity of Investigational Product

The vials of seed lots of strains PTL-ETEC-002 and PTL-ETEC-003 (100 vials per lot of each strain) were supplied by Acambis Research Limited. (100, Fulbourn Rd., Cambridge CB1 9PT, United Kingdom) in clear, neutral type 1 glass vials sealed with grey butyl rubber stoppers.

Secondary seed lots (100 cryovials per lot of each strain; 1 mL of suspension in a 2 mL cryovial) were prepared at Johns Hopkins University (JHU), standard operating procedure for secondary seed lot preparation and related FDA correspondence is provided in **Appendix**

12.7. The JHU lot numbers were 071498 (PTL-ETEC-002) and 070898 (PTL-ETEC-003). The seeds were stored at –70°C in a temperature-monitored secure freezer in the laboratories of the VTU in the Johns Hopkins Bloomberg School of Public Health and then transported to the GCRC of the Johns Hopkins Hospital for administration to the volunteers. The Principal Investigator (PI), A. Louis Bourgeois, PhD, MPH and George Gomes made the vaccine preps. The actual vaccine dose was determined by plate counts performed on the inoculum after volunteer dosing was completed. This was performed in the VTU in the Johns Hopkins Bloomberg School of Public Health. After dosing, all vaccine remaining on that day was inactivated by autoclaving.

Buffer solution was comprised of 200 ml of CeraVacx (a rice-based buffer containing 2 grams of sodium bicarbonate, 0.5 grams of trisodium citrate in addition to proprietary rice syrup).

6.6 Method of Assigning Subjects to Treatment Groups

Eligible subjects as they were screened were randomly assigned to one of the six groups to receive vaccine or placebo, two-dose regimen, on Day 0 and Day 10.

6.7 Selection of Doses in the Study

The dose of 2 x 10^9 CFU, two-dose regimen, was chosen for this study. The parent strain of PTL-ETEC-002 and PTL-ETEC-003, E1392/75/2A was previously tested by Levine et al.[8] as a live ETEC vaccine in two studies with doses ranging from 1 x 10^9 to 6 x 10^{10} bacteria. Diarrhea was seen as a side effect in approximately 10% of volunteers receiving doses greater than 1 x 10^{10} bacteria.

In the inpatient study, the vaccine strains at oral doses up to 5x10^9 CFU were associated with mild and moderate symptoms by protocol and/or case report form definition. Neither IgG nor IgA serum anti-CFA I antibody responses were detected in any of the volunteers. IgA Anti-CFA/II responses were seen in the ALS specimens from all volunteers given the highest doses of either vaccine strain[9]. Theses CFA/II specific ALS responses peaked on Day 7-post vaccination and returned to near baseline by Day 10-post vaccination.

These data suggest that an oral dose of 2x10^9 of either vaccine construct would be well tolerated and formed the basis for further evaluation of PTL-ETEC-002 and PTL-ETEC-003 in this outpatient study.

6.8 Selection and Timing of Dose for Each Subject

As described in **Section 7.1**, subjects were randomly assigned into one of six groups. Each comprised the oral administration of Buffer solution was

comprised of 200 ml of CeraVacx™ (a rice-based buffer containing 2 grams of sodium bicarbonate, 0.5 grams of trisodium citrate in addition to 7 grams of a proprietary rice syrup).

Volunteers were requested to fast for 90 minutes before and after administration of vaccine and were observed to ensure consumption of the entire contents of each vaccine.

6.9 Blinding

This was a double-blind study. Neither the volunteers nor the clinical and laboratory teams assessing the tolerability, shedding patterns and immunogenicity of either vaccine were aware of the specific group assignments. For the laboratory assess of fecal shedding and immunology. Anti-CFA/II specific immune responses following immunization were determined qualitatively, the criteria for determining positive and negative responders were determined prior to unblinding and the assignment of positive and negative responders was also performed on blinded data.

6.10 Prior and Concomitant Therapy

Prior ETEC vaccination at any time, or treatment with antibiotics within 7 days of vaccination were grounds for exclusion or dismissal from the study. Anti-pyretics were not permitted during the follow-up period unless discussed beforehand with study personnel. This was to avoid masking of any vaccine-induced fever.

6.11 Treatment Compliance

All vaccinations were conducted in the outpatient unit at Vaccine Testing Unit (VTU) at the Johns Hopkins University Bloomberg School of Public Health. A member of the VTU staff witnessed that the buffer and vaccine solutions were completely consumed, and documented on the vaccine accountability record.

6.12 Efficacy and Safety Variables

6.12.1 Efficacy, Immunogenicity and Safety Assessments

A direct assessment of efficacy (i.e. protection against ETEC) was not made or planned in this trial. It is anticipated that this will be carried out a later date using the most tolerable and immunogenic of the two vaccine constructs, either PTL-ETEC-002 or PTL-ETEC-003.

Antibody lymphocyte supernatant (ALS) assays

The antibody-secreting cell (ASC) response was determined in peripheral blood mononuclear lymphocytes (PBMLs) by comparing the number of ELISPOTS at baseline (Day 0) with results 7 days after

vaccine administration (Days 7 or 17). An ASC response was defined as 1.3 or more spots per 10^6 cells 7 days after vaccination and, if the baseline value was positive, a doubling of the baseline value. PBMLs were also analyzed by antibody lymphocyte supernatant assay (ALS). Unstimulated PBMLs were cultured for 48 hours and the culture supernatant subjected to ELISA assay for CFA/II specific IgG or IgA. An ALS response was defined as a 2-fold or greater increase compared to the baseline sample.

Serum antibody assays

Blood for measurement of anti-CFA/II IgA and IgG was obtained on Days 0, 7, 10, 17, 24, and 38. A response was defined as a 2-fold or greater increase compared to the antibody level on Day 0. For those receiving two doses of vaccine, responses on Days 7 and 10 were attributed to the first dose and responses after Day 10 were attributed to the second dose of vaccine.

The serum specimens were assayed by ELISA for IgA and IgG antibodies to the CFA/II antigen using antigen provided by Acambis Research Limited (Note: Acambis antigen was used to generate the data included in this report; similar assays have been performed with pure Cs1 and Cs3 antigen from NMRC but this data is not included in this report.) The assay was performed by pre-coating the plate with CFA/II antigen using a concentration of I μg/mL in PBS. After an overnight incubation at room temperature, the plates were blocked with BSA and washed. Three-fold dilutions of the volunteers' sera were prepared starting with a dilution of 1:10 in the first cup. The plates were incubated for one hour and subsequent to incubation with HRP-labelled anti-human IgG and substrate. Between each step, the plates were washed with PBS-Tween 20. The plates were read in an automatic ELISA reader where the wavelength was 450 nm.

To establish the appropriate concentration of CFA antigen for the assay, a validation study was carried out using varying dilutions of antigen, and sera from mice. Serum #I was from mice that had been immunized with a CFA/II bearing strain. Serum #2 was from mice immunized with an isogenic strain without CFA/II expression, and serum #3 was from mice that had not been immunized. Using these reagents, the titration results were similar when the concentration of the CFA/II varied from 5 to 45 μg/mL. There was a slight drop in Absorbance values when the antigen concentration was lowered to 1 μg, and a major drop when the concentration was lowered to 0.2 μg. The titers of the two immunized mice were higher than the serum from the non-immunized mice, but the serum from the mice immunized with CFA-negative *E. coli* was significantly higher than the non-immunized mouse serum, suggesting that the CFA antigen contained some antigens from the bacteria in addition to CFA antigen. The concentration of

1 μg/mL appeared to be optimal for differentiating the two immune mice sera.

A standard serum pool was developed as a positive control. To make the standard, 0.3 mL of sera collected on day 10 from those volunteers who had received the dose of 10^9 CFU were pooled. The test serum from each volunteer was tested on the same plate on the same day and a titration of the standard serum was included on each plate.

Specific Fecal IgA

Fecal samples for measurement of anti-CFA/II IgA was obtained on Days 0, 7, 10, 17 and 38. Although all the samples were run by ELISA, evaluable sets of samples were subjected to the following criteria: 1) The total IgA concentration must be at least 20 μg/mL fecal extract, 2) The total IgA concentration of the pair being evaluation must not exceed a 10-fold difference. A response was defined as a 2-fold or greater increase compared to the antibody level on Day 0. For those receiving two doses of vaccine, responses on Days 7 and 10 were attributed to the first dose and responses after Day 10 were attributed to the second dose of vaccine.

Safety

Safety was assessed by way of investigator and/or study staff assessment and review of documented signs and symptoms of illness. A symptom diary was maintained for seven (7) days after each dose. Subjects were also asked to return to the VTU for an assessment of adverse events prior to the first dose vaccination and Days 3, 7, and 10 after the first dose and Days 3, 7, 10, 14 and 28 after the second dose. Stool was collected on Days 3, 7 and 10 after the first dose and Days 3, 7, 10 14 and 28 after the second dose to detect the vaccine strain.

Colonization with either PTL-ETEC-002 or PTL-ETEC-003 ETEC strains was assessed by culture of stool specimens collected by the subject on Day 0, 3, 6-7, 10, 11-12, 16-18, 20-21, 24-25. For those subjects who continue to shed bacteria on Day 24-25 or beyond, specimens were collected and cultured until 2 negative stool cultures were obtained: on Day 33-36, 38, 41-43, and 53-54. For purposes of simplification, Day 6-7 will be referred to as Day 6, Day 11-12 as Day 11, Day 16-18 as Day 16, Day 20-21 as Day 20, Day 24-25 as Day 24, Day 33-36 as Day 33, Day 41-43 as Day 41, Day 53-54 as Day 53. Stool specimens were collected on days designated above and cultured on MacConkey agar and on MacConkey agar with streptomycin (Mac-strep). Colonies that grew on the Mac-strep plate were presumed to be vaccine strains and five colonies were spotted onto Luria agar, a complete growth medium, and onto minimal media (Turner et. al. 2001 Infect Immun.) Control (wild type) strains of *E. coli* grow on both of these agars, but the vaccine strains do not grow on the minimal media.

At least one colony of the vaccine strains was saved on nutrient agar slants. If the vaccine strain was still present in the stool 14 days after the second dose, Ciprofloxacin was to be administered (500 mg BID for 3 days) and at least two follow-up stool specimens were to be collected to verify that the vaccine strain has been cleared.

All stools were examined, graded and weighed by the nurse. The stool consistency was graded as 1=formed, 2=soft/mushy, 3=thick liquid, 4=opaque watery, 5=rice in water. Diarrhea was defined $\geq$ 3 unformed or liquid in a 24 hr. period (since this was an outpatient study we had to go with stool number versus volume as a determinant). Dysentery was defined as the occurrence of diarrhea with blood in the stool as detected as grossly visible blood.

A fever was defined as the occurrence of an oral temperature >38.0°C sustained in at least two occasions four hours apart. Where oral temperatures of ≥38.0°C were recorded on two occasions four hours apart, appropriate cultures were obtained and ciprofloxacin (500mg BID for 3 days) was prescribed. After completion of treatment, at least two follow-up stool specimens were to be collected to verify that the vaccine strain had been cleared.

For the assessment of reactogenicity, all signs and symptoms of grade 1 or more were reviewed. The general symptoms assessed included: Oral Temperature (>100.4°F), Felt ill, Weakness, Headache, Lightheadedness, Muscle Aches, and Chills. Gastrointestinal symptoms assessed included: Decreased Appetite, Gas, Cramping, Nausea, Abdominal Pain, Gurgling Stomach, Blood in Stool, Belching, Urgency of Defecation, Pain in the Rectum with Defecation, Vomiting, Unformed Stools, and Other. The symptoms were graded by volunteers (self-reported) on diary cards as 0=absent, 1=present, but does not impact on normal daily activity, 2=present and interferes with normal daily activity, 3=present and severe enough to prevent normal daily activity.

A serious adverse event (SAE) was defined as any untoward medical occurrence that at any dose: results in death, is life-threatening, requires or prolongs hospitalization, results in persistent or significant disability/incapacity, or was a congenital abnormality/birth defect. It was standard operating procedure of the VTU to provide preliminary information on the SAE to the Principal Investigator who reported the SAE to the FDA, JCCI IRB and Acambis Research Limited within 24 hours of the knowledge of such an event.

6.13 Appropriateness of Measurements

Other clinical studies with ETEC vaccine candidates[8,10,11,12] have indicated that